

Research article

A major genetic component of BSE susceptibility

Katrin Juling*¹, Hermann Schwarzenbacher¹, John L Williams^{2,3} and Ruedi Fries¹

Address: ¹Chair of Animal Breeding, Technical University of Munich, Hochfeldweg 1, 85354 Freising-Weihenstephan, Germany, ²Division of Genetics and Genomics, Roslin Institute, Roslin, Midlothian EH25 9PS, UK and ³CERSA, Parco Tecnologico Padano, Via Einstein 26900 Lodi, Italy

Email: Katrin Juling* - katrin.juling@tierzucht.tum.de; Hermann Schwarzenbacher - hermann.schwarzenbacher@tierzucht.tum.de; John L Williams - john.williams@tecnoparco.org; Ruedi Fries - ruedi.fries@tierzucht.tum.de

* Corresponding author

Published: 02 October 2006

Received: 15 May 2006

BMC Biology 2006, 4:33 doi:10.1186/1741-7007-4-33

Accepted: 02 October 2006

This article is available from: <http://www.biomedcentral.com/1741-7007/4/33>

© 2006 Juling et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Coding variants of the prion protein gene (*PRNP*) have been shown to be major determinants for the susceptibility to transmitted prion diseases in humans, mice and sheep. However, to date, the effects of polymorphisms in the coding and regulatory regions of bovine *PRNP* on bovine spongiform encephalopathy (BSE) susceptibility have been considered marginal or non-existent. Here we analysed two insertion/deletion (indel) polymorphisms in the regulatory region of bovine *PRNP* in BSE affected animals and controls of four independent cattle populations from UK and Germany.

Results: In the present report, we show that two previously reported 23- and 12-bp insertion/deletion (indel) polymorphisms in the regulatory region of bovine *PRNP* are strongly associated with BSE incidence in cattle. Genotyping of BSE-affected and control animals of UK Holstein, German Holstein, German Brown and German Fleckvieh breeds revealed a significant overrepresentation of the deletion alleles at both polymorphic sites in diseased animals ($P = 2.01 \times 10^{-3}$ and $P = 8.66 \times 10^{-5}$, respectively). The main effect on susceptibility is associated with the 12-bp indel polymorphism. Compared with non-carriers, heterozygous and homozygous carriers of the 12-bp deletion allele possess relatively higher risks of having BSE, ranging from 1.32 to 4.01 and 1.74 to 3.65 in the different breeds. These values correspond to population attributable risks ranging from 35% to 53%.

Conclusion: Our results demonstrate a substantial genetic *PRNP* associated component for BSE susceptibility in cattle. Although the BSE risk conferred by the deletion allele of the 12-bp indel in the regulatory region of *PRNP* is substantial, the main risk factor for BSE in cattle is environmental, i.e. exposure to feedstuffs contaminated with the infectious agent.

Background

Endogenous prion protein is known to play a central role in the pathogenesis of transmitted prion diseases [1,2]. The gene encoding the prion protein, *PRNP*, has therefore been suggested as a candidate locus for disease suscepti-

bility. In humans, mice and sheep, coding variants of *PRNP* have been shown to have major effects on the susceptibility to and incubation time of such diseases [3-8]. However, no clear relationships between bovine *PRNP* polymorphisms and susceptibility to bovine spongiform

encephalopathy (BSE) have been revealed so far [9-12]. Here we examine the effects of two insertion/deletion (indel) polymorphisms that were tentatively associated with BSE in a small case-control study [11]. Both the 23-bp indel [11] in the promoter region and the 12-bp indel [13] in intron 1 affect binding sites for transcription factors (RP58 and SP1, respectively), and thus might affect the expression of *PRNP* [14]. We analysed both polymorphisms in BSE-affected and control animals of four different populations: Holstein-Friesian cattle from the United Kingdom (UK Holstein) and Germany (German Holstein), German Brown and German Fleckvieh.

Results

Single marker analyses

Genotyping of both indel polymorphisms and χ^2 testing revealed significant association of each polymorphism with the BSE status in all populations except German Fleckvieh. Genotypes with deletion alleles and deletion alleles of both loci are generally overrepresented in the affected animals of all breeds (Table 1, Table 2 and Figure 1). Using Fisher's combined probability test, highly significant associations between the deletion allele and the BSE cases were found for both the 23-bp ($P = 2.01 \times 10^{-3}$) and the 12-bp indel ($P = 8.66 \times 10^{-5}$) over all populations. A combined logistic regression analysis involving all breeds also revealed highly significant associations between both indels and BSE ($P = 5.7 \times 10^{-5}$ and $P = 1.2 \times 10^{-7}$, respectively). Modelling the genotype data shows that the BSE risk tends to increase in line with the number of deletion alleles at both loci (Table 2). Heterozygous and homozygous carriers of the 12-bp deletion allele, when compared with non-carriers, have relatively higher risks of BSE, ranging from 1.32 to 4.01 and 1.74 to 3.65 in the different breeds. The corresponding population attributable risks [15,16], i.e. the portion of the BSE incidence in the population that is due to the risk allele (12-bp deletion allele), range from 0.35 to 0.53 (Table 2).

Haplotype analyses

We performed haplotype analysis, to estimate the combined effect of both polymorphisms. Strong linkage disequilibrium was present in all populations (e.g. in UK Holstein: $D' = 0.99$ C.I. = 0.96 – 1.0, LOD = 145.31; $r^2 = 0.745$), with the most frequent haplotypes being 23del-12del, 23ins-12ins and 23del-12ins. Haplotype 23ins-12del occurs in < 1% in all populations and was therefore excluded from further analyses. The χ^2 test indicated significant deviations from the expected haplotype frequencies in cases and controls of all populations except German Fleckvieh (Figure 2 and Table 3) and logistic regression modelling revealed significant contrasts in the ORs. The 23del-12del haplotype is associated with an increased risk of BSE incidence, whereas the 23ins-12ins haplotype seems to be protective. The effect of the 23del-

12ins haplotype depends on the population, but it does not differ significantly from that of the 23ins-12ins reference haplotype in any population. Additionally, the effect of the 23del-12del haplotype in the combined analysis is highly significantly different from that of the remaining major haplotypes ($P = 3.7 \times 10^{-6}$ and $P = 9.6 \times 10^{-4}$ for the 23ins-12ins and 23del-12ins haplotypes, respectively).

Diplo type analyses

We attempted to assess the risk of BSE infection associated with each diplo type. Five common diplo types were found (Figure 3 and Table 4), with the sixth, 23del-12ins/23del-12ins, being relatively rare (< 3 %); as such, it was excluded from further analyses. For German Holstein control animals, the diplo types were inferred from estimated haplo type frequencies (see Additional File 1: Inferring allele, genotype, haplo type and diplo type frequencies from half-sibs). In both Holstein populations, the 23del-12del/23del-12del diplo type is significantly overrepresented in affected animals as revealed by χ^2 testing ($P = 2.5 \times 10^{-3}$ and $P = 9.2 \times 10^{-3}$ for UK and German Holstein, respectively). Significant association was also observed in the German Brown breed, where the 23ins-12ins/23ins-12ins diplo type is highly overrepresented in the controls ($P = 4.7 \times 10^{-4}$), and the 23del-12del/23ins-12ins diplo type occurs significantly more often in the cases ($P = 6.0 \times 10^{-4}$). Analysis over all populations indicates that the highest risk is conferred by the 23del-12del/23del-12del diplo type (Table 4). Figure 4 shows the diplo type conferred ORs obtained from logistic regression analysis of the combined data, accounting for population effects, and the data of each individual population. The ORs were calculated using the absolute risks averaged across all five diplo types as reference in order to allow for a meaningful comparison of populations. The analyses of the individual populations and the combined analysis suggest that risk of BSE in a population tends to increase in line with the number of deletion alleles at the 12-bp indel.

Modelling the modes of allelic action and relative contribution of both polymorphisms

Likelihood ratio tests were applied as proposed by North et al. [17] to investigate both the modes of allelic action for both loci (additive versus dominance effects) and for possible epistatic effects (additive by additive, additive by dominance and dominance by dominance interactions). The results indicate that for the individual populations as well as for the combined analysis neither the inclusion of dominance effects nor inclusion of epistatic effects leads to a significantly better fit compared to a model that accounts for additive effects only. Hence, likelihood ratio tests were performed to verify whether fitting an additive effect for either indel locus reduces the likelihood significantly compared to a full model that includes additive effects of both polymorphisms. This analysis revealed that

Table 1: Summary of genotype association analyses with BSE status

UK Holstein	23-bp indel Frequency of Genotypes					12-bp indel Frequency of Genotypes				
	Cases ^a	(n)	Controls ^a	(n)	P-Value ^c	Cases ^a	(n)	Controls ^a	(n)	P-Value ^c
del/del	55.4	(201)	46.4	(128)	2.4×10^{-2}	49.4	(173)	37.0	(100)	2.1×10^{-3}
del/ins	41.0	(149)	48.9	(135)	4.7×10^{-2}	45.4	(159)	51.9	(140)	1.1×10^{-1}
ins/ins	3.6	(13)	4.7	(13)	4.7×10^{-1}	5.1	(18)	11.1	(30)	5.8×10^{-3}
German Holstein^b										
del/del	45.7	(58)	38.0 ^b	(119) ^b	1.4×10^{-1}	40.0	(50)	28.2 ^b	(87) ^b	1.6×10^{-2}
del/ins	46.5	(59)	47.3 ^b	(148) ^b	8.8×10^{-1}	45.6	(57)	49.8 ^b	(154) ^b	4.2×10^{-1}
ins/ins	7.9	(10)	14.7 ^b	(46) ^b	5.2×10^{-2}	14.4	(18)	22.0 ^b	(68) ^b	7.2×10^{-2}
German Brown										
del/del	20.9	(9)	13.8	(12)	2.9×10^{-1}	7.0	(3)	3.3	(3)	3.4×10^{-1}
del/ins	65.1	(28)	41.4	(36)	1.1×10^{-2}	51.2	(22)	22.2	(20)	7.8×10^{-4}
ins/ins	14.0	(6)	44.8	(39)	5.0×10^{-4}	41.9	(18)	74.4	(67)	2.5×10^{-4}
German Fleckvieh										
del/del	53.8	(57)	46.3	(63)	2.5×10^{-1}	45.3	(48)	39.4	(54)	3.6×10^{-1}
del/ins	39.6	(42)	43.4	(59)	5.6×10^{-1}	46.2	(49)	45.3	(62)	8.8×10^{-1}
ins/ins	6.6	(7)	10.3	(14)	3.1×10^{-1}	8.5	(9)	15.3	(21)	1.1×10^{-1}

^a Represents frequency (%) of genotypes; (n) indicates the number of genotypes.

^b Control consists of half-sibs (see Additional File 1: Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs).

^c P-values of χ^2 -test against the both remaining genotypes

exclusion of the 23-bp indel does not reduce the likelihood significantly in any of the individual populations or in the combined data. However, exclusion of the 12-bp indel polymorphism reduces the likelihood significantly in UK Holstein ($P = 1.04 \times 10^{-3}$) and in the combined

analysis ($P = 6.89 \times 10^{-4}$). Thus, the main effect on BSE susceptibility seems to result from the 12-bp indel. This is supported by the haplotype analysis, which showed that the effect of the 23del-12ins haplotype does not differ significantly from that of 23ins-12ins, whereas the 23del-

Table 2: Summary of single marker association analyses with BSE status

23-bp indel	Frequency of Deletion				P-Value ^c	Logistic Regression Analysis			
	Cases ^a	(n)	Controls ^a	(n)		P-Value ^d	OR ins/del ^e	OR del/del ^e	PAR ^f
UK Holstein	75.9	(363)	70.8	(276)	4.2×10^{-2}	2.9×10^{-2}	1.36	1.84	0.24
German Holstein	68.9	(127)	61.8 ^b	(335)	4.3×10^{-2}	4.4×10^{-2}	1.38	1.91	0.46
German Brown	53.5	(43)	34.5	(87)	3.3×10^{-3}	5.4×10^{-3}	4.74	4.62	0.69
German Fleckvieh	73.6	(106)	68.0	(136)	1.8×10^{-1}	1.9×10^{-1}	1.31	1.71	0.36
Combined		(639)		(834)	2.0×10^{-3} ^g	5.7×10^{-5}	1.43	2.04	
12-bp indel									
UK Holstein	72.1	(350)	63.0	(270)	5.8×10^{-4}	3.2×10^{-4}	1.61	2.60	0.53
German Holstein	62.8	(125)	53.2 ^b	(357)	9.1×10^{-3}	1.1×10^{-2}	1.48	2.20	0.35
German Brown	32.6	(43)	14.4	(90)	5.9×10^{-4}	1.1×10^{-2}	4.01	3.65	0.44
German Fleckvieh	68.4	(106)	62.0	(137)	1.5×10^{-1}	1.5×10^{-1}	1.32	1.74	0.44
Combined		(624)		(854)	8.7×10^{-5} ^g	1.2×10^{-7}	1.58	2.50	

^a Represents frequency (%) of deletion allele as calculated from genotypes; (n) indicates the number of individuals.

^b Control consists of half-sibs (see Additional File 1: Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs).

^c P-values of χ^2 -test.

^d P-values from logistic regression, modelling the number of deletion alleles on BSE status.

^e Odds Ratios with reference genotype: ins/ins.

^f Population Attributable Risk: the portion of the BSE incidence in the population that is due to 12-bp deletion allele [15, 16].

^g P-Values of Fisher's Combined Probability test.

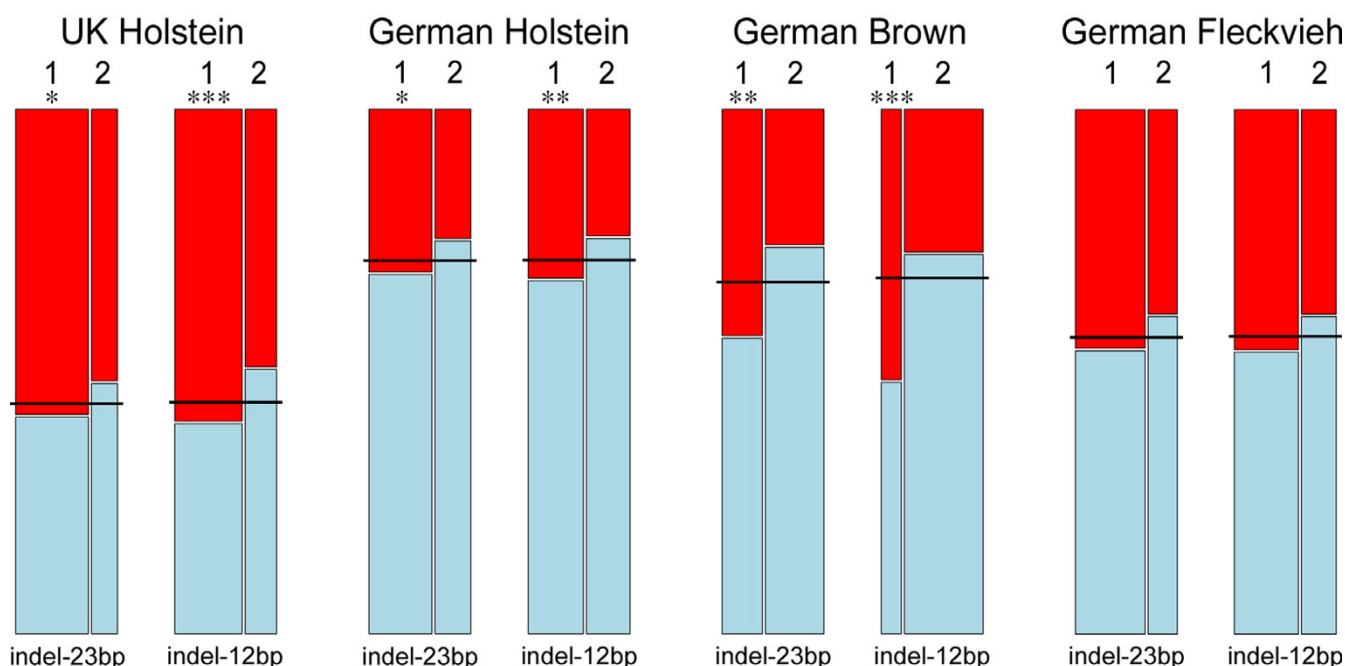


Figure 1

Allele Frequencies. Allele frequencies of the 23- and 12-bp insertion/deletion polymorphisms in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light-blue) animals. "1" and "2" above the columns represent the deletion and insertion alleles, respectively. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the allele frequency. Asterisks indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

12del haplotype is significantly associated with higher BSE risk in the Holstein populations and in German Brown (Table 3).

Discussion

We found significant association between *PRNP* promoter indel polymorphisms and the BSE status in three cattle populations. The association is not significant in the German Fleckvieh population. However, as in the other populations, the deletion alleles are overrepresented in BSE-affected animals of German Fleckvieh. Furthermore, including the German Fleckvieh population into the combined analyses leads to more significant overall association (data not shown). Thus, there is no reason to assume a fundamentally different situation in German Fleckvieh with regard to the effect of the indel polymorphisms on BSE susceptibility. Haplotype analysis and likelihood ratio tests identified that the main effect on BSE susceptibility is due to the 12-bp indel. Although suggestive, our findings cannot directly verify a causal role for either or both indel polymorphisms in the aetiology of BSE. The observed associations could result from another, genetic variant in linkage disequilibrium with the indel polymorphisms, although a functional role for these two polymorphisms is supported by their location in regulatory regions of *PRNP*. *In vitro* analyses have shown that the 12-

bp deletion disrupts binding of the SP1 transcription factor and *in vivo* and *in vitro* investigations suggest that the two polymorphisms affect *PRNP* expression, albeit with the direction of the effects remaining to be clarified [14]. One could speculate that, all other factors being equal, susceptibility to BSE might be reduced with a lowered expression of endogenous prion protein. Elevated expression of the prion protein, in contrast, would result in more substrate for conversion into the pathogenic form and possibly higher BSE susceptibility.

The calculated population attributable risks allow assessing the effect of the indel polymorphisms on the BSE epidemic. For example, 53% and 35% of the Holstein BSE cases in UK and Germany, respectively, can be explained by the 12-bp deletion allele (Table 2). Thus, if the UK Holstein population had been fixed for the alternative 12-bp insertion allele (and a possible effect on time to onset is not considered), roughly 80,000 BSE cases would have occurred instead of the 184,000 cases that were recorded. Similarly, the number of cases in German Holstein would have been reduced from 138 to about 90. Although the two populations have similar frequencies of the risk increasing deletion alleles, the BSE-incidence is orders of magnitude smaller in the German than in the UK population, which is most likely explained by differences in envi-

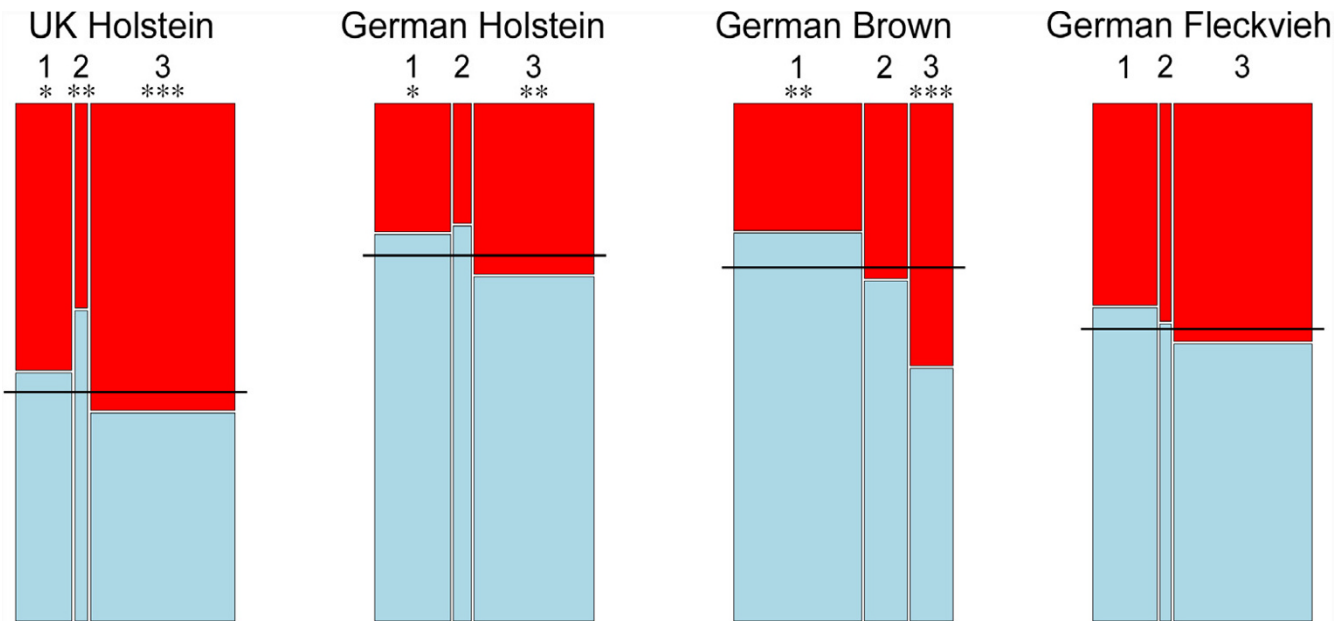


Figure 2
Haplotype Frequencies. Haplotype frequencies in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light blue) animals. Numbers above the columns represent the haplotypes: (1) 23ins-12ins, (2) 23del-12ins and (3) 23del-12del. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the haplotype frequency. Stars indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

ronmental exposure, such as a generally less intensive use of meat and bone meal in ruminant nutrition in Germany. Thus, although the indel polymorphisms – and particularly the 12-bp indel – are associated with the BSE risk, they are not the main risk factors. Instead, the decisive factor is environmental: that is to say, whether or not

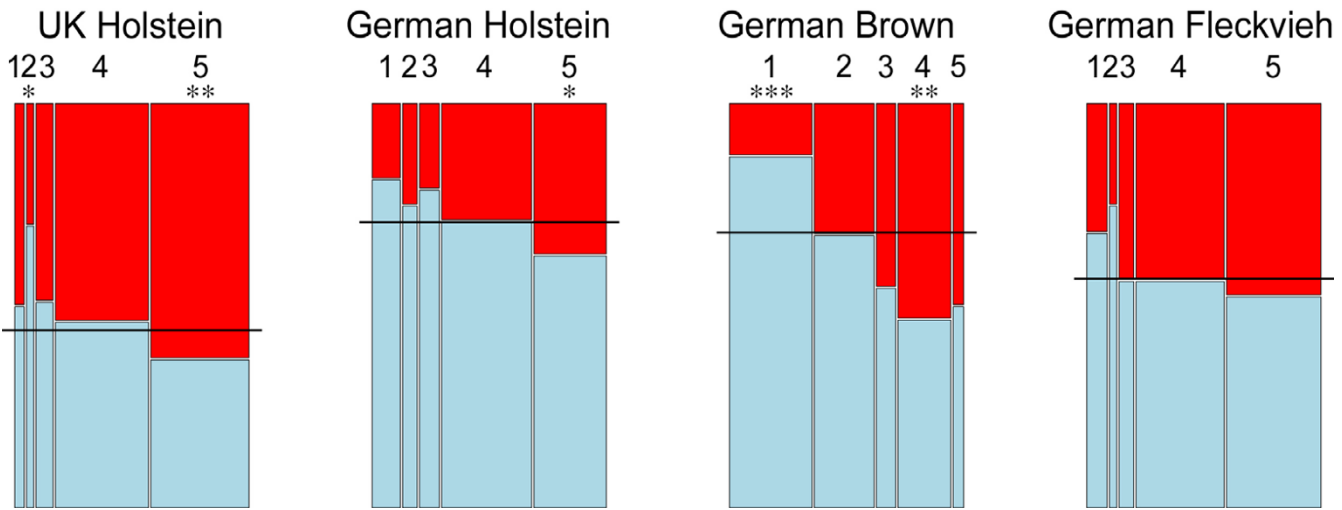


Figure 3
Diplotype Frequencies. Diplotype frequencies in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light blue) animals. Numbers above the columns represent the haplotypes: (1) 23ins-12ins/23ins-12ins, (2) 23ins-12ins/23del-12ins, (3) 23del-12ins/23del-12del, (4) 23ins-12ins/23del-12del and (5) 23del-12del/23del-12del. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the diplotype frequency. Asterisks indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

Table 3: Summary of haplotype association analyses with BSE status

UK Holstein	Frequency of Haplotypes				Logistic Regression Analysis			
	Cases ^a	(n)	Controls ^a	(n)	P-Value ^c	OR ^d	P-Value 23ins-12ins ^e	P-Value 23del-12ins ^f
23ins-12ins	24.2	(171)	29.1	(159)	5.1×10^{-2}	1		6.6×10^{-2}
23del-12ins	4.1	(29)	8.1	(44)	3.1×10^{-3}	0.62	6.6×10^{-2}	
23del-12del	71.7	(506)	62.8	(343)	8.9×10^{-4}	1.37	1.6×10^{-2}	1.3×10^{-3}
German Holstein								
23ins-12ins	30.2	(77)	39.1 ^b	(232)	1.4×10^{-2}	1		8.1×10^{-1}
23del-12ins	7.2	(17)	9.1 ^b	(56)	3.9×10^{-1}	0.93	8.1×10^{-1}	
23del-12del	62.2	(162)	51.3 ^b	(327)	7.5×10^{-3}	1.49	1.4×10^{-2}	1.1×10^{-1}
German Brown								
23ins-12ins	45.0	(39)	66.1	(119)	1.3×10^{-3}	1		1.8×10^{-1}
23del-12ins	22.5	(18)	19.4	(35)	5.7×10^{-1}	1.58	1.8×10^{-1}	
23del-12del	32.5	(27)	14.4	(26)	7.8×10^{-4}	3.14	5.4×10^{-4}	8.4×10^{-2}
German Fleckvieh								
23ins-12ins	26.8	(58)	32.6	(90)	1.7×10^{-1}	1		7.5×10^{-1}
23del-12ins	5.1	(11)	5.4	(15)	8.7×10^{-1}	1.15	7.5×10^{-1}	
23del-12del	68.1	(147)	62.0	(171)	1.7×10^{-1}	1.33	1.5×10^{-1}	7.2×10^{-1}
Combined								
23ins-12ins						1		5.0×10^{-1}
23del-12ins						0.90	5.0×10^{-1}	
23del-12del						1.50	3.7×10^{-6}	9.6×10^{-4}

^a Represent frequency (%) of haplotypes; (n) indicates the number of haplotypes.

^b Control consists of half-sibs (see Additional File 1: Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs).

^c P-values from χ^2 -test against pooled observations of all remaining haplotypes.

^d Odds Ratios with reference haplotype 23ins-12ins.

^e P-values from logistic regression, testing the risk effect compared to reference haplotype 23ins-12ins.

^f P-values from logistic regression, testing the risk effect compared to reference haplotype 23del-12ins.

an animal is sufficiently exposed to the infectious agent. Eliminating or minimising exposure to the infectious agent should therefore be considered to be the primary measure to reduce BSE incidence, even in the light of the strong genetic component of BSE susceptibility as we report it here. However, selective breeding to reduce the frequency of the susceptibility alleles would still bring an additional protective component that should not be discounted.

Conclusion

In this study we presented significant association between *PRNP* promoter indel polymorphisms and the BSE status in three of four investigated cattle populations (Figure 1, Table 1 and Table 2). We conclude that the main effect on BSE susceptibility seems to result from the 12-bp indel. The causality of either or both polymorphism cannot be verified in this study, however our data highlight that the 12 bp deletion allele is associated with increased risk for an animal to succumb to feed-borne BSE (Table 4 and Figure 4). Thus, our findings show a substantial genetic component for the susceptibility of cattle to BSE, conferred by

variants in the regulatory region of *PRNP*, a gene that has been shown to be involved in prion disease susceptibility/resistance in other species. However, the different dimensions of the BSE epidemic in UK and Germany, on the one hand, and the similar frequencies of the *PRNP*-associated susceptibility alleles in UK and German cattle, on the other, indicate, that the main BSE risk factor for cattle is environmental, i.e. exposure to contaminated feed, and not genetic.

Methods

Subjects and controls

The BSE status of German animals was diagnosed in clinically-suspect animals and by testing all slaughtered animals 24 months of age or older in certified local laboratories based on the presence of proteinase K resistant prion protein fragments. The initial diagnosis had to be confirmed by the German National BSE Reference Laboratory at the Friedrich-Löffler Institute (Riems Island, Germany). If suitable tissue was available, the diagnosis was histopathologically confirmed. DNA from German BSE affected animals was extracted by the Friedrich-Löffler

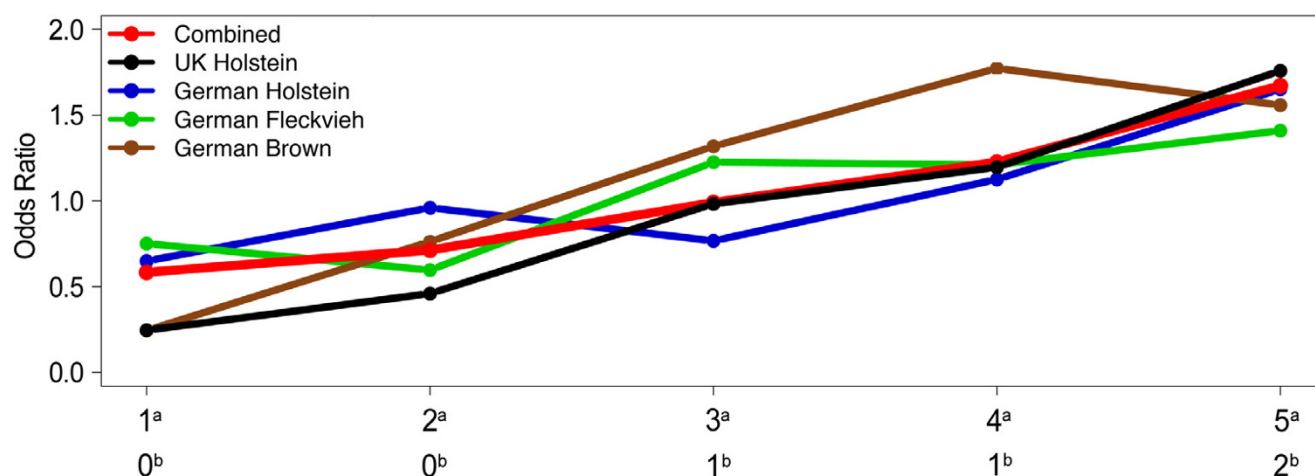


Figure 4

Odds Ratios of Diplotypes. Odds ratios (ORs) conferred by the diplotypes in each population and over all populations. The ORs were calculated using the absolute risks averaged across all five diplotypes as reference. ^aNumbers refer to diplotypes: (1) 23ins-12ins/23ins-12ins, (2) 23ins-12ins/23del-12ins, (3) 23del-12ins/23del-12del, (4) 23ins-12ins/23del-12del and (5) 23del-12del/23del-12del. ^bNumbers indicate number of deletion alleles at the 12-bp indel locus.

Institute using the QiAamp DNA Mini Kit (Qiagen Valencia, CA, USA) after tissue decontamination with 13.5 M guanidine chloride. A total of 276 cases collected from November 2000 until the end of 2005 were available. Based on the farmer's declaration, 127 of these cases were of German Holstein, 106 were of German Fleckvieh and 43 were of the German Brown breed. Control animals for the German Holstein case group consisted of 627 paternal half-sibs that were approximately contemporaneous with the BSE animals and had geographically similarly distributions. Only the maternally-inherited alleles were considered (see Additional File 1: Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs). Control animals ($n = 90$) for German Brown cases were selected from bulls used for artificial insemination such that their pedigree was representative for the German Brown population. The German Fleckvieh control group ($n = 137$) consisted of bulls kept at the experimental Station Hirschau of the Technical University of Munich. All were purchased at markets throughout Bavaria and can be considered to be representative for the German Fleckvieh population. Samples of BSE-affected UK animals were collected between 1990 and 1993. Animals were identified initially as BSE suspects by veterinary diagnosis of live animals, with the initial diagnosis being confirmed by histopathological examination following culling. Blood samples were obtained from a total of 365 BSE affected and 276 BSE unaffected age-matched paternal half-sib offspring that were born on the same farms in the same birth cohorts as the BSE-affected animals. The proportion of affected to unaffected animals was similar in each of the

37 half-sib groups. None of the controls were subsequently identified as BSE cases in the database of the UK Department for Environment, Food and Rural Affairs (Defra). DNA was obtained from blood via proteinase K digestion and phenol-extraction.

Genotyping

Sequence information was obtained from GenBank accession number [AJ298878](#). UK and German Holstein animals were genotyped by single base extension reactions (iPLEX™ Assay, SEQUENOM™, San Diego, CA, USA), with the first base of the insertion and the first base after the insertion representing the insertion and deletion alleles, respectively. Alleles were detected by MALDI-TOF mass spectrometry (MassARRAY®, SEQUENOM™, San Diego, CA, USA). German Brown and German Fleckvieh case and control animals were genotyped using PCR, followed by high resolution agarose (3%) gel electrophoresis to visualise the allelic PCR products. PCR primer 5'-CCTGTT-GAGCGTGCTCGT-3' and 5'-ACCTGCGGCTCCTCTACC-3' were used for genotyping the 23-bp indel (191bp/168bp) and primer 5'-GGAAGTCACGTGAAGGCACT-3' and 5'-CAAAGAGTTGGACAGGCACA-3' for the 12-bp indel (215bp/203bp).

Statistical analyses

Statistical analyses were performed using the R software environment [18], extended by the packages genetics (version 1.2.0) and logistf (version 1.05) [19]. Haploview 3.2 [20] based on the expectation maximisation (EM) method was used to infer linkage disequilibrium and

Table 4: Summary of diplotype association analyses with BSE status

UK Holstein	No. of 12-bp Deletion	Frequency of Diplotypes				P-Value ^d	OR ^e
		Cases ^a	(n)	Controls ^a	(n)		
23del-12ins/23del-12ins	0	0	(0)	<1.0	(3)	-	
23ins-12ins/23ins-12ins ^c	0	3.7	(13)	4.8	(13)	4.8×10^{-1}	1 ^c
23del-12ins/23ins-12ins	0	1.7	(6)	5.1	(14)	1.4×10^{-2}	0.45
23del-12ins/23del-12del	1	6.5	(23)	8.8	(24)	2.7×10^{-1}	0.96
23ins-12ins/23del-12del	1	39.0	(139)	43.6	(119)	2.4×10^{-1}	1.17
23del-12del/23del-12del	2	48.7	(172)	36.6	(100)	3.6×10^{-3}	1.72
German Holstein							
23del-12ins/23del-12ins	0	<1.0	(1)	<1.0	(3) ^b	-	
23ins-12ins/23ins-12ins ^c	0	7.8	(10)	15.3	(47) ^b	6.1×10^{-2}	1 ^c
23del-12ins/23ins-12ins	0	5.5	(7)	7.1	(22) ^b	6.0×10^{-1}	1.48
23del-12ins/23del-12del	1	6.3	(8)	9.4	(29) ^b	2.4×10^{-1}	1.18
23ins-12ins/23del-12del	1	39.1	(50)	40.6	(125) ^b	3.4×10^{-2}	1.73
23del-12del/23del-12del	2	40.6	(52)	26.6	(82) ^b	1.2×10^{-2}	2.54
German Brown							
23del-12ins/23del-12ins	0	2.4	(1)	3.3	(3)	-	
23ins-12ins/23ins-12ins ^c	0	14.3	(6)	45.6	(41)	3.7×10^{-4}	1 ^c
23del-12ins/23ins-12ins	0	26.2	(11)	25.6	(23)	9.6×10^{-1}	3.12
23del-12ins/23del-12del	1	11.9	(5)	6.7	(6)	3.1×10^{-1}	5.40
23ins-12ins/23del-12del	1	38.1	(16)	15.6	(14)	4.3×10^{-3}	7.27
23del-12del/23del-12del	2	7.1	(3)	3.3	(3)	-	6.38
German Fleckvieh							
23del-12ins/23del-12ins	0	<1.0	(1)	0	(0)	-	
23ins-12ins/23ins-12ins ^c	0	6.5	(7)	10.9	(15)	2.4×10^{-1}	1 ^c
23del-12ins/23ins-12ins	0	1.9	(2)	4.3	(6)	-	0.79
23del-12ins/23del-12del	1	6.5	(7)	6.5	(9)	1	1.63
23ins-12ins/23del-12del	1	38.9	(42)	39.1	(54)	1	1.61
23del-12del/23del-12del	2	45.4	(49)	39.1	(54)	2.9×10^{-1}	1.88
Combined							
23del-12ins/23del-12ins	0						
23ins-12ins/23ins-12ins ^c	0						1 ^c
23del-12ins/23ins-12ins	0						1.22
23del-12ins/23del-12del	1						1.70
23ins-12ins/23del-12del	1						2.10
23del-12del/23del-12del	2						2.86

^a Represents the frequency (%) of diplotypes; (n) indicates the number of individuals.

^b Calculated assuming Hardy-Weinberg equilibrium from haplotype frequency estimates (see Additional File 1: Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs).

^c Diplotype taken as reference.

^d P-values from χ^2 -test against pooled observations of all remaining diplotypes.

^e Odds Ratios from logistic regression.

Phase 2.1.1 [21,22], which applies a Bayesian statistical framework, was used to derive haplotypes and individual diplotypes. Diplotypes with posterior probabilities lower than 0.9 were excluded from further analysis. For the calculation of population attributable risks, population genotype frequencies were inferred by weighting frequency estimates obtained for cases and based on the average annual incidence rate of BSE (UK: 0.2 %, Germany: 0.0012 % [23]).

Authors' contributions

KJ conceived the study and participated in its design, set up DNA samples, carried out the genotyping, performed the data analysis, participated in the statistical analysis and prepared the manuscript. HS performed statistical analyses and participated in study design and helped to draft the manuscript. JLW organised the collection of the UK samples, participated in the study design and helped to draft the manuscript. RF coordinated the project and

participated in the study design and in drafting the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Inferring allele, genotype, haplotype and diplotype frequencies from half-sibs. Supplementary methods: Control animals for the German Holstein case group consisted of paternal half-sibs. Details on inferring the maternally inherited alleles.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1741-7007-4-33-S1.pdf>]

Acknowledgements

We thank M. Groschup and U. Ziegler of the Friedrich-Löffler Institute for Novel and Emerging Infectious Diseases on Riems Island for providing DNA samples of German BSE cases and F. Conraths of the Friedrich-Löffler Institute of Epidemiology at Wusterhausen for breed information. DNA of German Holstein animals was kindly provided by the Institute of Animal Breeding of the Christian-Albrechts-University of Kiel (E. Kalm), the Department of Animal Breeding and Genetics of the Justus-Liebig-University of Gießen (G. Erhardt) and the Molecular Biology Unit of the Research Institute for the Biology of Farm Animals, Dummerstorf (C. Kühn). D. Matthews of the Veterinary Laboratories Agency Weybridge, formally from MAFF, is acknowledged for facilitating the collection of the UK samples, which was funded by various MAFF projects. We thank T. Meitinger and P. Lichtner from Institute of Human Genetics of GSF National Research Center for Environment and Health, Neuherberg, for advice in the setup of genotyping assays and for providing access to the genotyping facility. Thanks are also due to Olaf Bininda-Emonds for careful English language editing. This study was carried out within the framework of FORPRION and was supported by the Bavarian Ministry of Health, Food and Consumer Protection.

References

- Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C: **Mice devoid of PrP are resistant to scrapie.** *Cell* 1993, **73**:1339-1347.
- Hunter N: **Molecular biology and genetics of bovine spongiform encephalopathy.** In *The Genetics of Cattle* Edited by: Fries R, Ruvinsky A. Wallingford: CABI Publishing; 1999:229-246.
- Windl O, Dempster M, Estibeiro JP, Lathe R, de Silva R, Esmonde T, Will R, Springbett A, Campbell TA, Sidle KC, et al.: **Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic analysis of predisposing mutations and allelic variation in the PRNP gene.** *Hum Genet* 1996, **98**:259-264.
- Collinge J, Beck J, Campbell T, Estibeiro K, Will RG: **Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease.** *Lancet* 1996, **348**:56.
- Carlson GA, Goodman PA, Lovett M, Taylor BA, Marshall ST, Peterson-Torchia M, Westaway D, Prusiner SB: **Genetics and polymorphism of the mouse prion gene complex: control of scrapie incubation time.** *Mol Cell Biol* 1988, **8**:5528-5540.
- Hunter N, Cairns D, Foster JD, Smith G, Goldmann W, Donnelly K: **Is scrapie solely a genetic disease?** *Nature* 1997, **386**:137.
- Palmer MS, Dryden AJ, Hughes JT, Collinge J: **Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease.** *Nature* 1991, **352**:340-342.
- Mead S, Stumpf MP, Whitfield J, Beck JA, Poulter M, Campbell T, Uphill JB, Goldstein D, Alpers M, Fisher EM, et al.: **Balancing selection at the prion protein gene consistent with prehistoric kurulike epidemics.** *Science* 2003, **300**:640-643.
- Hunter N, Goldmann W, Smith G, Hope J: **Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland.** *Vet Rec* 1994, **135**:400-403.
- Neibergs HL, Ryan AM, Womack JE, Spooner RL, Williams JL: **Poly-morphism analysis of the prion gene in BSE-affected and unaffected cattle.** *Anim Genet* 1994, **25**:313-317.
- Sander P, Hamann H, Pfeiffer I, Wemheuer W, Brenig B, Groschup MH, Ziegler U, Distl O, Leeb T: **Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds.** *Neurogenetics* 2004, **5**:19-25.
- Nakamitsu S, Miyazawa T, Horiuchi M, Onoe S, Ohoba Y, Kitagawa H, Ishiguro N: **Sequence variation of bovine prion protein gene in Japanese cattle (Holstein and Japanese Black).** *J Vet Med Sci* 2006, **68**:27-33.
- Hills D, Comincini S, Schlaepfer J, Dolf G, Ferretti L, Williams JL: **Complete genomic sequence of the bovine prion gene (PRNP) and polymorphism in its promoter region.** *Anim Genet* 2001, **32**:231-232.
- Sander P, Hamann H, Drogemüller C, Kashkevich K, Schiebel K, Leeb T: **Bovine prion protein gene (PRNP) promoter polymorphisms modulate PRNP expression and may be responsible for differences in bovine spongiform encephalopathy susceptibility.** *J Biol Chem* 2005, **280**:37408-37414.
- Greenland S, Rothman KJ: **Measures of Effects and Measures of Association.** In *Modern Epidemiology* 2nd edition. Edited by: Greenland S, Rothman KJ. Philadelphia: Lippincott Williams & Wilkins; 1998:47-64.
- Greenland S: **Applications of Stratified Analysis Methods.** In *Modern Epidemiology* Edited by: Greenland S, Rothman KJ. Philadelphia: Lippincott Williams & Wilkins; 1998:281-300.
- North BV, Curtis D, Sham PC: **Application of logistic regression to case-control association studies involving two causative loci.** *Hum Hered* 2005, **59**:79-87.
- The R Project for Statistical Computing** [<http://www.r-project.org/>]
- The Comprehensive R Archive Network** [<http://cran.r-project.org/>]
- Barrett JC, Fry B, Maller J, Daly MJ: **Haploview: analysis and visualization of LD and haplotype maps.** *Bioinformatics* 2005, **21**:263-265.
- Stephens M, Smith NJ, Donnelly P: **A new statistical method for haplotype reconstruction from population data.** *Am J Hum Genet* 2001, **68**:978-989.
- Stephens M, Donnelly P: **A comparison of bayesian methods for haplotype reconstruction from population genotype data.** *Am J Hum Genet* 2003, **73**:1162-1169.
- World Organisation of Animal Health – Bovine Spongiform Encephalopathy (BSE)** [http://www.oie.int/eng/info/en_esb.htm]

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

